

## ***Strongylocentrotus purpuratus* 96-Hour Larval Development Test**

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### **1.0 OBJECTIVE**

The purpose of the development test with the sea urchin, *Strongylocentrotus purpuratus*, is to determine if seawater, interstitial water, sea surface microlayer, or other samples cause abnormal development of exposed embryos relative to embryos exposed to control or reference samples (US EPA 1995). The test may also be used to determine the concentration of a test substance that causes abnormal development.

### **2.0 EQUIPMENT**

The following equipment is necessary to conduct the toxicity test at the Marine Pollution Studies Laboratory at Granite Canyon (MPSL). The word "clean" here and throughout this procedure means that the item has been cleaned according to the MPSL glassware cleaning procedures outlined in a separate SOP (MPSL SOP 1.3).

#### **2.1 Urchin Collection and Culture**

- Tanks, trays, or aquaria for holding adult sea urchins
- Air pump, airlines, and air stones -- for aerating water containing adult urchins (for static systems and emergency aeration for flow-through systems).

#### **2.2 Test Initiation**

- Squirt bottle filled with seawater
- 20-mL glass scintillation vials (leached in dilution water for 24 hours)
- 10-mL glass pipettes and pipettor
- Environmental chamber ( $15 \pm 1^{\circ}\text{C}$ , ambient laboratory illumination for 16 hours/day)
- Culture micropipettors and tips (10-1000 mL)
- Glass Pasteur pipettes and bulb
- Glacial acetic acid
- Hemacytometer
- Sedgwick-Rafter counting cell
- Culture graduated cylinders
- 0.5 M KCl
- Syringe and 24-gauge needle for injecting urchins
- Control water (Granite Canyon seawater) and hypersaline brine

#### **2.3 Test Termination**

- Inverted compound microscope
- Data sheets
- Formaldehyde, 37% (concentrated buffered formalin) and volumetric pump
- Fume hood

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- Counter

## **2.4 Water Quality**

- Meters, probes and spectrophotometer for measuring pH, dissolved oxygen, salinity, and ammonia
- Thermometers (glass spirit thermometer and logger)
- Graduated pipettes and 30-ml glass vials
- Gloves and appropriate safety gear (see MPSL lab safety manual)

## **2.5 Dilution Water**

Dilution water consists of ambient Granite Canyon seawater, filtered to 1  $\mu\text{m}$ , at ambient salinity (33-34‰). This water is used to prepare eggs and sperm for toxicity tests, and for diluting test solutions.

## **3.0 EXPERIMENTAL DESIGN**

Aquatic toxicity tests can be used as screening tools or as part of more comprehensive studies to assess water quality. Careful consideration must be given to site characteristics, reference site selection, field replication, choice of synoptic measures, seasonal factors, and comprehensive planning and peer review to determine that study designs are adequate to meet program objectives.

This laboratory toxicity test consists of five replicate scintillation vials for each sample. Samples are occasionally diluted into several concentrations. Vials are arranged randomly, and each receives 250 urchin embryos. The quality of test embryos and testing conditions is determined through concurrent testing of reference toxicants (positive controls), seawater control, and brine control (negative controls). Testing of reference sites is recommended to demonstrate the suitability of interstitial waters in the absence of toxic contaminant concentrations. Dissolved oxygen, pH, salinity, ammonia, and temperature are measured at the beginning and end of the exposure.

## **4.0 SAMPLE PREPARATION**

Label scintillation vials as indicated on the randomization sheet generated for the test. Label another set of vials, three each with the sample number of each sample, to be used for water quality measurements at the end of the test. Determine the salinity of the test solutions. Be sure to stir all samples before measuring salinity, and measure salinity immediately after stirring. Samples salinities below 32‰ are adjusted to 34‰ using hypersaline brine made from frozen seawater or artificial salts. Check the pH of brine. If necessary, adjust the brine pH by adding acid or sodium hydroxide until it is between 7.5-8.5.

Using the random number sheet, aliquot 10 mL of sample to scintillation vials and water quality containers. Minimize sample exposure to sunlight (never leave samples in direct sunlight), and schedule loading times to avoid prolonged sample exposure to temperatures above 15°C.

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### **5.0 CONTROLS**

#### **5.1 Seawater and Brine Controls (Negative Controls)**

A seawater control consisting of 1- $\mu$ m filtered Granite Canyon water should accompany each batch of samples. If any salinity adjustments were made a brine controls must also be prepared. The brine control must contain the same amount of brine as the lowest salinity sample.

#### **5.2 Reference Toxicant Tests (Positive Controls)**

Conduct a concurrent reference toxicant test each time an urchin development test is conducted (standard procedure). The reference toxicant test similar exposure time, and provides data on the relative sensitivity of each batch of urchin embryos.

Conduct a concurrent reference toxicant test with every new batch of organisms. The reference toxicant test is for a similar exposure time, and provides data on the relative sensitivity of each batch of urchin gametes. Prepare a stock solution of 10,000  $\mu$ g Cu/L by weighing 0.0268 grams of copper chloride ( $\text{CuCl}_2$ ), and pouring the weighed solid into one liter of distilled water in a plastic volumetric flask. Reference toxicant solutions should be four replicates of 0, 3.2, 5.6, 10, 18, and 32  $\mu$ g/L. Other concentrations may be added between these if greater precision is desired for quality control chart purposes. Prepare concentrations according to dilution schedule. Start with the control solutions and progress to the highest concentration to minimize contamination. Use plastic volumetric flasks and beakers to reduce loss of copper to container walls.

### **6.0 TEST INITIATION**

#### **6.1 Test Organisms**

Newly fertilized embryos of the sea urchin, *Strongylocentrotus purpuratus* are used in the development toxicity test. Animals may be collected in the field or obtained from a commercial supplier. *S. purpuratus* is distinguished from *S. franciscanus*, a less common cogener, by it's purple to occasionally green color. Urchins can be maintained easily in aquaria or other tanks provided with running seawater. Urchins will eat a wide variety of marine algae, but prefer giant brown kelp, *Macrocystis pyrifera*. To ensure year-round spawning, broodstock are held at ambient seawater temperature, in complete darkness. Tanks should be cleaned weekly during feeding, and ambient salinity should be maintained. The sex of the urchins may be determined at the time of spawning. Once these animals are re-conditioned they may be spawned for use in later toxicity tests.

#### **6.2 Test System**

Five replicates are used for all treatments. Each replicate contains 10 mL of test solution. A brine control is necessary if any salinity adjustments are required. The brine control should contain a volume of brine equal to the

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brine volume used to adjust the sample with the lowest salinity. Make the brine control by first adding enough fresh Nanopure water to adjust Granite Canyon dilution water to a salinity equal to that of the lowest salinity sample. Then adjust this sample back up to  $34 \pm 2\text{‰}$  using an appropriate volume of brine or artificial salts (see salinity adjustment data sheet).

## **6.3 Collection and Preparation of Gametes**

To perform the test repeatedly, quality gametes must first be collected, and then diluted to the appropriate density of embryos for addition to the test vials.

### **6.3.1 Spawning of Urchins**

- Place ten urchins on several layers of paper towels on a clean surface.
- Inject each urchin with 0.5 mL of 0.5 M KCl in the soft tissue surrounding the Aristotle's lantern and gently shake the animals once or twice to stimulate gamete release.
- Re-inject with 0.5 mL of 0.5 M KCl, any urchins that have not spawned after 10 minutes.
- Females release orange-colored eggs, and males release cream-colored sperm.
- Collect gametes before 30 minutes have elapsed. Sperm must be used within 4 hours.

### **6.3.2 Gamete Quality**

- Eggs must be inspected for uniformity and roundness. Select only females with uniformly round eggs that lack follicles. Do not use a batch of eggs if a high proportion of germinal vesicles are present or if eggs are released from females in large clumps.
- Eggs and sperm should be washed off the urchins with a squirt bottle filled with 1- $\mu\text{m}$  filtered seawater. Gametes should be rinsed into separate beakers. Use special care to avoid cross contamination between sperm and eggs.
- Place one drop of eggs onto a well slide and add a small amount of sperm to test fertilization. Check for a fertilization membrane. If no fertilization membrane is present, isolate new eggs.
- Place the eggs in the constant temperature room (15°C) until ready for counting.

### **6.3.6 Fertilization of Gametes**

Use the urchin spawning worksheet to determine the appropriate sperm and egg dilution factors. Sperm and eggs are combined based on worksheet equations (500:1 sperm to egg ratio). Embryos are added at a density of 250 ( $\pm 10\%$ ) per vial. Fertilization should be  $>90\%$  and embryos should be added within one hour of fertilization.

## **7.0 TEST TERMINATION**

To terminate the test, use a toxic dispenser or disposable pipette, add 1.0 mL 37 % buffered formalin to each sample to give a final formalin concentration of 4%. Gently shake containers to mix. As an alternate fixative, 0.5 mL of

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1.0 % glutaraldehyde may be added to each test container. Test containers may now be capped and stored for later evaluation.

## **8.0 DATA COLLECTION AND TABULATION**

Count a minimum of 100 individuals (e.g., embryos, plutei, etc.) per sample using hand counter with multiple keys. Use one key to indicate normal larvae and another to indicate abnormal larvae. Due to the shape of the larvae, their orientation may need to be adjusted in order to determine normality.

### **8.1 Normal Larvae**

Normally developed larvae have the following characteristics:

- Four spicules that extend at least half the distance from the base to the apex.
- A gut that demonstrates signs of differentiating into three chambers. In some cases it is not possible to distinguish all three chambers of the gut. If the apex of the gut appears lobed and constricts distally, then normal gut development may be inferred.

### **8.2 Abnormal Larvae**

Abnormal or inhibited larvae may fit into any one of the following categories:

- Pathological pre-hatched: unfertilized eggs and fertilized eggs with the membrane still visible.
- Pathological hatched: larvae that have no fertilization membrane and demonstrate an extensive degree of malformation or necrosis. Most larvae appear as dark balls of cells or disassociated blobs.
- Retarded: larvae at blastula and gastrula stages that have no gut differentiation or have underdeveloped spicules.
- Gut abnormalities: larvae that are otherwise normal with exogastrulated guts, lacking differentiation or lacking guts.
- Skeletal abnormalities: misshapen larvae as a result of missing spicules or underdeveloped spicules, rods growing in abnormal directions or extraneous spicules.

Note: Spicules that are slightly separated at the apex might be caused by formalization and should not be considered abnormal.

Calculate Percent Normal Development for each control replicate test. Test acceptability is 80% normal development in the reference test seawater control.

## **9.0 DATA HANDLING AND TEST ACCEPTABILITY**

Immediately after test termination, check the data sheet to determine whether dilution water and salinity controls have acceptable normal development ( $\geq 80\%$ ). Tests with temperature, salinity, or dissolved oxygen measurements

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outside the specified ranges, may be considered conditionally acceptable based on the project officer's best professional judgment. Acceptable temperatures are  $15 \pm 1^\circ\text{C}$ ; acceptable dissolved oxygen concentration is 60-100% saturation.

## **10.0 REFERENCES**

U.S. Environmental Protection Agency. 1995. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to west coast marine and estuarine organisms. Office of Research and Development. EPA/600/R-95/136. August 1995

## **11.0 TEST SUMMARY**

Species:	<i>Strongylocentrotus purpuratus</i>
Test Duration:	96 hours
Endpoint:	Normal Development
Organism Source:	Wild-caught adult broodstock that has been maintained in the laboratory in constant darkness and free flowing seawater
Test Salinity:	Ambient $\pm 2\text{‰}$
Test Temperature:	$15 \pm 1^\circ\text{C}$
Dilution water:	1 $\mu\text{m}$ filtered seawater
Lighting:	16:8 light:dark
Replication:	5 replicates per sample, plus three for water quality
Test Containers:	20 mL glass scintillation vials
Loading:	Approximately 250 embryos per vial in 10 mL
Water Quality:	Dissolved oxygen, pH, salinity, ammonia, and temperature
Reference Toxicant:	Copper Chloride ( $\text{CuCl}_2$ )
Acceptability Criteria:	Controls: $\geq 80\%$